Effect of synthetic pyrethroids on expression of human ortholog neurodegenerative genes in *Caenorhabditis elegans*

SYNOPSIS

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1.0 INTRODUCTION

Dysfunctioning of the neuronal signaling pathway due to the misfolding and aggregation of specific protein results neuron degeneration (Dimitriadi and Hart, 2010). Neuron degeneration causes the progressive loss of the structure and function of the neurons that causes the neuronal death (Dimitriadi and Hart, 2010; Deshmukh *et al.,* 2012). Alzheimer's, Parkinson's, Huntington's and Amyotrophic lateral sclerosis are the most leading neurodegenerative diseases those occur as a result of neurodegenerative processes. Of these disorders Parkinson's is the one of the most leading movement disorder, characterized by motor and non motor dysfunction.

The Motor dysfunction includes rhythmic shaking, involuntary movement (tremor at rest), slowness of movement (Brady kinesia), increased muscle tone (Rigidity), difficulty in communication, weight loss and memory impairment (Eeden *et al.,* 2003; Uversky, 2004; Nass *et al.,* 2008; Leung *et al.,* 2008) while non motor dysfunction includes dementia, depression and sleeping disorders (Jankovic, 2008). The Parkinson's disease is more common in the rural areas and the use of pesticides and herbicides are increases the risk of this disease (Freire and Koifman, 2012). The genetic studies suggest that the death of cells in Parkinson's disease occur as a result of oxidative stress, mitochondrial dysfunction, protein aggregation and proteosome dysregulation (Dawson *et al.,* 2003).

To study neurodegenerative disorders, the researchers now turned to the soil nematode, *C.elegans* (Dimitriadi and Hart, 2010). *C.elegans* was originally chosen in 1963 by Sydney Brenner as a model organism to explore the genetics of developmental biology, physiology, behavior and the nervous system (Culetto and Sattelle, 2000; Palop and Mucke, 2010). Since then *C.elegans* has been utilized to identify and characterize the molecular components involved in the *in vivo* assessment of the human disease (Kaletta and Hengartner, 2006; Leung *et al.,* 2008; Markaki and Tavernaraskis, 2010).

C.elegans also has a number of properties due to which it is used as model organism:

- The *C.elegans* is a free living soil nematode with small body size (~1 mm in length), short life cycle (~3 days), and high reproducible rate and has short lifespan (Brenner, 1974; Culetto and Sattelle, 2000).
- Transparent body allows visualization of organs and neurons *in vivo* (Boyd *et al*., 2010).
- The adult *C.elegans* hermaphrodite contains 302 neurons and their position and identity remain fixed from animal to animal (White *et al*., 1986).
- Fully sequenced genome has 42% genes of the human related diseases those are ortholog in *C.elegans* (Culetto and Sattelle, 2000; Dimitriadi and Hart, 2010).
- *C.elegans* also has neurotransmitters and associated receptors, found in higher eukaryotes including dopamine, acetylcholine, Gama-amino butyric acid (GABA), and serotonin (Boyd *et al*., 2010).

The *C.elegans* is sensitive to the human neuroreactive compounds, these compounds interact with the human Orthologues targets present in the worm. This suggests that compounds that inhibit the dopamine neuronal cell death in the *C.elegans* will likely be cause the Parkinson's disease in human (Nass *et al.*, 2008). Parkinson's disease is caused due to the degeneration of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) (Nass *et al.*, 2001; Bear *et al.*, 2007; Harrington *et al.*, 2010; Nuytemans *et al.*, 2010; Zhou *et al.*, 2013).

Orthologues of genes linked to Parkinson's disease exist in the *C.elegans* including Parkin (Pdr-1), PINK 1(Pink-1), DJ-1 (Djr-1) and LRRK2 (lrk-1) (Springer, 2005; Lim and Ng, 2009; Dimitriadi and Hart, 2010; Vistbakka *et al*., 2012). Mutations in the pdr-1, pink-1, djr-1, lrk-1 genes causes the degeneration of dopaminergic neurons in *C.elegans* (Leung *et al.*, 2008; Dimitriadi and Hart, 2010; Nuytemans *et al.*, 2010; Wolozin *et al.*, 2011; Vistbakka *et al.*, 2012). *C.elegans* have four bilaterally symmetrical pairs of dopamine neurons, two pairs of CEP (Cephalic dendrites) and one pair of ADE (Anterior dendrites) within the head and one pair of PDE (Posterior dendrites) in the posterior lateral position (Nass *et al.*, 2001). Over the last decades the *C.elegans* also gains popularity in toxicological research as a model organism, to investigate the neurological effects of pesticides induced toxicity

(Vinuela *et al.*, 2010; McVey, 2012). Earlier studies shows that a number of neurodegenerative diseases are caused due to the effect of pesticides, like Huntington's and Parkinson's caused by Rotenone, Parkinson's also caused by Paraquat and Maneb and Alzheimer's caused by Propoxur (Population health branch, 2002, Sherer *et al.*, 2003, Uversky, 2004, Jadiya *et al.*, 2012).

All of these above mentioned diseases are caused due to the different mode of actions of pesticides, as Alzheimer's caused due to the inhibition of acetylcholine neurotransmitter and Parkinson's is caused due to inhibition of dopamine neurotransmitter (Uversky, 2004; Elwan *et al.*, 2006; Leung *et al.*, 2008; Dimitriadi and Hart, 2010; Wolozin *et al.*, 2011; Chemical watch Factsheet, 2011). It was revealed through the literature that most of the commercially available products used to control pests such as Hit, All out, Good night etc. contained the pesticides (Allethrin, Resmethrin, Prallethrin, Cyfluthrin, Bifenthrin and Transfluthrin) as a major component and these all pesticides belongs to the class Pyrethroid (Chemical Watch Factsheet; Fishel, 2014). Spray of the pesticides containing products releases tiny aerosol droplets and these droplets stay in the air and kill the pests on contact as well as humans also inhaled these aerosol with breathing that may affect the nervous system (Schleier and Peterson, 2011).

Present study aimed to see the effect of pesticides on the neurodegenerative ortholog genes in the *C.elegans* leading to Parkinson's disease due to the degeneration of dopamine neuron. For the present study we have selected class pyrethroid, because before to mention the specific one pesticide, screening is required on the basis of behavior and developmental level. The change in the behavior and development are the two indicator of nervous system that is affected by any adverse conditions (Dimitriadi and Hart, 2010). For the screening of the pesticides from pyrethroid class, Allethrin, Resmethrin, Prallethrin, Cyfluthrin, Bifenthrin and Transfluthrin are selected, as these are the most commonly used pesticides in the commercially available products. Further the neurotoxic effect of the selected pesticide will be checked on the degeneration of the dopaminergic neurons in *C.elegans* due to the mutation in ortholog gene pdr-1, pink-1, djr-1, lrk-1 in *C.elegans* leads to Parkinson's disease.

This work will explore the neurotoxic effect of synthetic pyrethroids pesticides used in the commercially available products to control pests, to which we are getting exposed either directly or indirectly that is the leading cause of Parkinson's disease. Synthetic pyrethroids are pesticides derived from naturally occurring pyrethrins, taken from pyrethrum of dried Chrysanthemum flowers. All selected pesticides are synthetic pyrethroids. The technical approaches which we are going to use in the present work have a complete profile as it includes physiological as well as genomic aspect to understanding the synthetic pyrethroid pesticides toxicities. This study also supports the National Toxicological Program's (NTPs) mission to evaluate the chemicals of public health concerns, to see the effect on human neurodevelopment.

2.0 REVIEW OF LITRETURE

3.0 2.1 NEURODEGENERATIVE DISEASE

The Alzheimer's disease is the first most common neurodegenerative disease and is firstly described by **Alois Alzheimer** in **(1907)** because of this it named Alzheimer's. The Parkinson's disease is the second most common neurodegenerative disease National Institute of Environmental Health Sciences **(NIEHS).** The 95% of Parkinson's disease cases showed that there is no genetic inheritance and this termed as sporadic while the remaining 5% are genetically inherited **(Dauer and Przedborski, 2003).** Toxicity in rotenone models on Parkinson's disease **(Sherer *et al.*, 2003).**

Parkinson's is a disease in which the nigrostriatal dopaminergic pathway becomes affected and the different epidemiological studies describe that there is an association between the pesticides exposure and incidence of Parkinson's disease **(Elwan *et al.*, 2006).** When the dopamine neurons become degenerated this supports the evidence that animals lack ability to respond to environmental changes **(Qin and Wheeler, 2007).** Acetylcholine is the major neurotransmitter in *C.elegans* which directly or indirectly regulates egg laying **(Bany *et al.*, 2003)** and locomotion **(Mullen *et al.*, 2007). Dimitriadi and Hart (2010)** described that the neurodegenerative diseases impose a burden on the society this underlying mechanism is neuronal dysfunction. The *C.elegans* has Dopamine, acetylcholine, y-amino butyric acid, and serotonin are the neurotransmitters and associated receptors those are also found in

higher eukaryotes this studies done by the **Boyd *et al.*, (2010)**. **Wolozin *et al.*, (2011)** described that the neurodegenerative disease such as Amyotrophic lateral sclerosis, Parkinson's, Alzheimer's, Huntington's are the disease is caused due to the degeneration of neurons and these diseases are incurable. *C.elegans* used as model organism for the molecular conservation for neuronal signaling pathways in vertebrate and invertebrate species so the researchers started using nematode *C.elegans* to study neurodegenerative diseases **(Wolozin *et al.*, 2011**).

To see the expression of stress genes in transgenic *C.elegans* model of Parkinson's disease by checking the effect of various pesticides **(Jadiya *et al.*, 2012).** The inhibition of tau accumulation in the model of tauopathy mitigates the proteotoxicity of *C.elegans* was studied by **Fatouros *et al.*, (2012). Alexander *et al.*, (2014)** explore that The *C.elegans* is advantageous in modeling the learning and memory impairments seen during the Alzheimer's disease. The *in vivo* observation of biomolecules and the automated analysis of protein aggregation used *C.elegans* as model organism for the neurodegenerative disease amyotrophic lateral sclerosis (ALS) was studied by **Cornaglia M. *et al.*, (2016). Song and Kim (2016)** found that Degeneration of dopaminergic neurons due to metabolic alterations and this alteration causes Parkinson's disease.

2.2 MODEL ORGANISM *Caenorhabditis elegans*

Brenner (**1974)** observed that the nematode *C.elegans* has small body size of ~1 mm is the length with a short and rapid life cycle (3 days). **Brenner** in (**1974)** explain that how the *C. elegans* can be grown in in vitro condition using NGM media and *E.coli* OP50 as food source. The hermaphrodite nematode *C.elegans* has 302 neurons and thus position and identity of the neurons remain store from animal to animal stated by **White *et al.*, (1986).** *C.elegans* was the first multicultural organism that has completely sequenced genome **(*C.elegans* sequencing Consortium, 1998)**. 42% genes those are related to human disease are ortholog of *C.elegans* **(Culetto and Sattelle, 2000**). **Alberts *et al.*, (2002)** has described that the hermaphrodite considered being a form of self fertile female and cross fertilization

after transfer of male sperm by mating and the hermaphrodites produce all the sperm in the L4 stage and then produce only oocytes and this has both male and female sex organs.

Emmons SW. (**2005)** described that the male contains the 473 neurons. The maintenance of the model organism *C.elegans* has described by **Steiernagle** in **(2006). Kaletta and Hengartner, (2006) and Leung MCK *et al.*, (2008)** described that the genes and the signaling pathways are well conserved between *C.elegans* and humans. The nematode *C.elegans* is an important animal model in the various fields like neurobiology, genetics and developmental biology **(Leung *et al.*, 2008).** The stress tolerance and the extension in the lifespan due to the effect of Reserpine by the use of *C.elegans* as an animal model **(Srivastava *et al.*, 2008).** The single hermaphrodite gives birth to a large number of offspring about ~300 those mature at four larval stages of L1-L4 **(Markaki and Tavernarakis, 2010). Boyd *et al.*,** (**2010)** described that *C.elegans* has transparent body that allows visualization of all cells and organs and the *C.elegans* also used in thoroughly studying in terms of its genetics, cell biology, behavior and development. The major neurotransmitter systems which allow the study of neurodegenerative diseases that help to the neuroscientists discovered by **(Dimitriadi and Hart**, **2010**). The hermaphrodite *C.elegans* possesses a tapered and pointed tail, whereas a male *C.elegans* possesses blunt tail and hermaphrodite includes a mouth, pharynx, intestine, gonad and they have neither a circulatory nor a respiratory system. **Boyd *et al.*,** (**2012)** studied that *C.elegans* reproduce quickly and produce large numbers of offspring and this will be easily maintained in a Petri dish. **McVey *et al.*, (2012)** described the neurotoxicity of pesticides used *C.elegans* as an emerging model system. *C.elegans* also used to detect the soil toxicity in Saudi Arabia studied by **Alhewairini S. *et al.*, (2016).**

2.3 PESTICIDES

The pyrethroids are also used to kill mosquitoes, cockroaches, fleas and other insects in the agriculture and commercially available products such as Allethrin, Resemthrin, cyfluthrin, transfluthrin, and Bifenthrin and the Allethrin was first synthesized by Milton S. Schechter in 1949 in the United States **(Pesticides information profile, 1993).** The pesticides those are acetylcholinestrase inhibitor reduced the tail thrashing and overall movement in the nematode and it also a modulators neurotransmitter that

has role in the locomotion, egg laying, pharyngeal pumping, defecation cycling and male mating **(Richmond and Jorgensen, 1999).**

The neurodegenerative disease may be caused by different ways as due to the effect of mutation in the gene and the pesticides also one of the reasons that responsible for the neuron degeneration like rotenone causes Huntington's disease **(Population health branch, 2002**). The pesticide rotenone causing the accumulation of the alpha synuclein and degenerate the dopamine neurons by the subcutaneous exposure of this pesticides **(Sherer TB *et al.*, 2002).** Neuronal protein activity is modulated by the ethanol, so to identify the mechanisms those are responsible for the intoxication screen the *C.elegans* mutants in response to ethanol altered behavior and this altered behavior include the loss of social inhibition and sedation this study was done by **Davies *et al.*, (2003).** The *C.elegans* used to examine the mechanism of toxicity of pesticides. Organophosphate has increased markedly since 1970s, when many insecticides such as DDT were banned. Over 80% pesticides poisoning caused by organophosphate and carbamate in the US **(Debra, 2004). Elwan *et al.*, (2006)** studied the alterations in the dopamine transporter function those are induced by the pyrethroid pesticides. Pyrethroids are the neurotoxin pesticides able to inhibit the transfer of the nerve impulses by blocking the receptor of acetylcholine discovered by **Krzepilko and Swiecilo, (2007).** Pyrethrin is the purified form of pyrethrum and it is a collection of six ester found within the pyrethrum extract **(Duchon *et al.*, 2009). (Yadav *et al.*, 2010)** has studied the cognitive function impairment and inhibition of acetylcholinestrase due to the exposure of propoxur attenuation by *Withania somnifera*.

Vinuela *et al.*, (2010) described gene expression analysis in response to organophosphate pesticides according to this the organophosphate pesticides affect the nervous system by the inhibition of enzyme acetylcholinestrase a regulator for the acetylcholine neurotransmitter. Propoxur was first registered by Bayer, AG in 1963 and it used to kill ticks, wasps and crickets was described in the **Chemical watch Factsheet, (2011).** Exposure of Organophosphorous pesticides on neurobehavioral development of Zebrafish and characterization of developmental switch between two cation chloride co transporters zfnkcc1 and zfkcc2 describe by **Yen J.H. (2012). Jadia *et al.*, (2012)** studied the expression level of stress related genes in the transgenic *C.elegans* model of neurodegenerative disease like Parkinson's

disease in response to various classes of pesticides. **Zhou *et al*.,** (**2013)** described that the exposure of environmental chemicals causes the neuron degeneration and the mitochondrial suppression in the model organisms of *Caenorhabditis elegans*. India used 234 registers pesticides are WHO class 1a pesticides, 15 are WHO class 1b pesticides, and 76 are WHO class 2nd pesticides **(India for Safe Food.htm).** The toxicity studies done with *C.elegans* are used chemicals as toxicants or environmental pollutants as a research tool. Many acetylcholinestrase inhibiting pesticides used to describe the function of molecular components of the cholinergic nervous system **(Delaware health and social services).** The development of the small and transparent *C.elegans* is reproducible so that the cell at any position of the body has the same lineage in the every individual **(Moy *et al.,* 2015).** Pesticide deltamethrin causes the changes in dopaminergic gene expression, neurochemistry and also in locomotory activity by using zebrafish as model organism discovered by **Kung *et al*., (2015).** The accumulation of acetylcholinestrase due to the exposure of organophosphate pesticides increases the risk of Alzheimer's disease **(Yadav *et al*., 2016).**

2.4 GENE EXPRESSION

The gene expression analysis provides a baseline for future studies and by this process the information on gene is used in the functional gene product and gene expression will be confirmed may be by using the technique quantitative real time PCR **(Link *et al.,* 2002)**. This technique requires the least amount of RNA and produces the largest amount of data in a short period of time and the total RNA prepared from the staged population by the use of the acid phenol method (Trizol) **(Link *et al*., 2002).** The technique Qualitative RT-PCR is used to measure the transcriptional level RNA of a gene **(Radoni *et al.,* 2003).** The scientist done work on reverse transcription and polymerase chain reaction and describes the principle and the dentistry **(Dos *et al*., 2004).**

The *C.elegans* used as a model organism to study the distinct patterns of gene and the protein expression elicited by the organophosphorous pesticides this study done by **Lewis *et al.,* (2009).** Gene expression profiling of substantia nigra dopaminergic neurons: insights into the Parkinson's disease

studied done by **Simunovic *et al.*, (2009)**. Genetic architecture of gene expression is complex. The gene expression phenotypes are associated with the genome with their location itself **(Romanoski *et al.*, 2010).** Genetic etiology of Parkinson's disease associated with mutations in PARK2, PINK1, LRRK2, and PARK7 genes done by **Nuytemans *et al.* (2010)**. Genetic architecture of gene expression is complex **(Rodriguez, 2011).**

4.0 OBJECTIVES

The objectives of our study are as follows:

1. To setup and maintenance of the culture of *C.elegans*.
2. *In vivo* assessment of behavioral changes in *C.elegans* due to the effect of synthetic Pyrethroids.
3. *In vivo* assessment of developmental changes in *C.elegans* due to the effect of synthetic Pyrethroids.
4. To study the degeneration of dopamine neurons due to the effect of synthetic Pyrethroid.
5. To Study the effect on the gene expression of human ortholog neurodegenerative genes in *C.elegans* due to the exposure of synthetic Pyrethroids.

5.0 MATERIALS AND METHODS

4.1 MATERIAL

For the present study to check the toxicity the wild type *C.elegans* N2 strains will be used. Culture will be procured from the *Caenorhabditis elegans* Genetics Center (University of Minnesota, Minneapolis, MN, USA) and the *E.coli* OP50 lawn will be used as a bacterial food source for *C.elegans* will also be procured from *C.elegans* Genetics Center (University of Minnesota, Minneapolis, MN, USA) (Stiernagle, 2006). Resmethrin, Prallethrin, Cyfluthrin, Bifenthrin and Transfluthrin are selected from Synthetic pyrethroids for the present study for screening because these are the most commonly found pesticides in commercially available products.

4.2 METHOD

The methodology of the above mentioned objectives are as follows:

4.2.1 CULTURE AND MAINTENANCE OF *C.elegans* AND PESTICIDE TREATMENT

C.elegans will be cultured on Nematode Growth Media (NGM) agar and seeded with an *E.coli* OP50 as a bacterial food source at 20℃. The culture of *E.coli* OP50 will be prepared in LB broth after incubating at 37℃ and stocks will be maintained at 4℃ that can be used for several months. L1 and L2 stage of *C.elegans* can be frozen and stored indefinitely in liquid nitrogen at -80℃ after adding glycerol to the freezing media (Brenner, 1974).

Treatment of the pesticides to the worm will be done in the following way. Pesticide treatment will begin from the synchronized parental generation. Synchronized L1 stage worms will be transferred on NGM plates, pre coated with pesticides in DMSO to achieve a desired concentration, and then the worm will be analyzed for the behavioral, developmental and neuronal changes (Wu *et al.,* 2014).

4.2.2 BEHAVIORAL CHANGES IN *C.elegans*

The alteration in the behavioral aspects is the indicator of the direct effect on nervous system. Behavioral changes will be studied by seeing the effect on Locomotion, learning, Egg laying, and reproductive behavior of *C.elegans* after the exposure of pesticides.

4.2.2.1 Locomotion

Locomotory behavior may be evaluated by head thrash and the body bends method following the methodology of Xue and Yang, 2016. The results of locomotory behavior will be further analyzed by using the softwares may be like Nemo, tracking software, Neurotracker 2.0 and track worm softwares.

4.2.2.2 Learning

Learning behavior will be examined by making the mazes made up of silicon rubber on the NGM agar plate following the methodology of Qin and Wheeler, (2006).

4.2.2.3 Egg laying

Egg-laying will be counted on NGM agar plates using copper ring method. Relative egg-laying frequency will be determined be taking an average of treated egg laying/ untreated egg laying (Davies *et al.,* 2003).

4.2.2.4 Reproductive behavior

One adult worm will be placed in each 1-ml test solution and incubated for three days (72 h) the number of offspring at all stages beyond the eggs will be counted to show the reproductive behavior (Middendorf and Dusenbery, 1993; Dhawan *et al.*, 1999).

4.2.3 DEVELOPMENTAL CHANGES IN *C.elegans*

Developmental changes will be evaluated by seeing the effect on Growth, Mortality, Paralysis and lethality.

4.2.3.1 Growth

Change in the growth of *C.elegans* will be confirmed by measuring the body length, before and after exposure of the pesticides with a microscope using a scaled lens (Roh *et al.,* 2007).

4.2.3.2 Mortality

Toxicity of pesticide on *C.elegans* will be studied by using the mortality as an endpoint. To check the mortality we will use the different lethal concentration of pesticides. The culture plate of *C.elegans* containing the test solution will be exposed at 20^0C. The worms will be declared dead if not giving a response by probing with platinum wire. Visualization will be done under dissecting microscope (Roh *et al.,* 2007).

4.2.3.3 Paralysis

For the paralysis assay, the worms will be monitored every day and will consider paralyse when they did not move even after repeated ridding (Cohen *et al.,* 2006) and the results of paralysis will be further analyzed by using the software may be like a parallel worm tracker.

4.2.3.4 Lethality

Lethality will be evaluated by calculating percent of surviving animals. After exposure, inactive worms will be counted under a dissecting microscope and judged to be dead if they did not respond to the stimulus given by a small metal wire (Xue and Yang, 2016).

4.2.4 NEURON DEGENERATION IN *C.elegans*

Effect on the dopaminergic neuron (CEP, ADE and PDE) due to the exposure of pesticide in *C.elegans* will be examined under a dissecting fluorescence microscope. The exposed worms will be analyzed by immobilizing with 2% of sodium azide, pre-dropped on 2% agarose. If the GFP in any part of the 2 pairs of cephalic dendrites (CEPs), 1 pair of anterior dendrites (ADE) and 1 pair of posterior dendrites (PDE) neuron find missing then the worms will be scored positive for neuron degeneration (Nass *et al*., 2001). The results of neuron degeneration will be further analyzed by using the softwares may be like neurite tracer, genesis and Wis neuromath.

4.2.5 GENE EXPRESSION

Gene expression of the pdr-1, pink-1, djr-1, lrk-2 genes after the pesticides treatment analysis will be done by two steps RT-PCR.

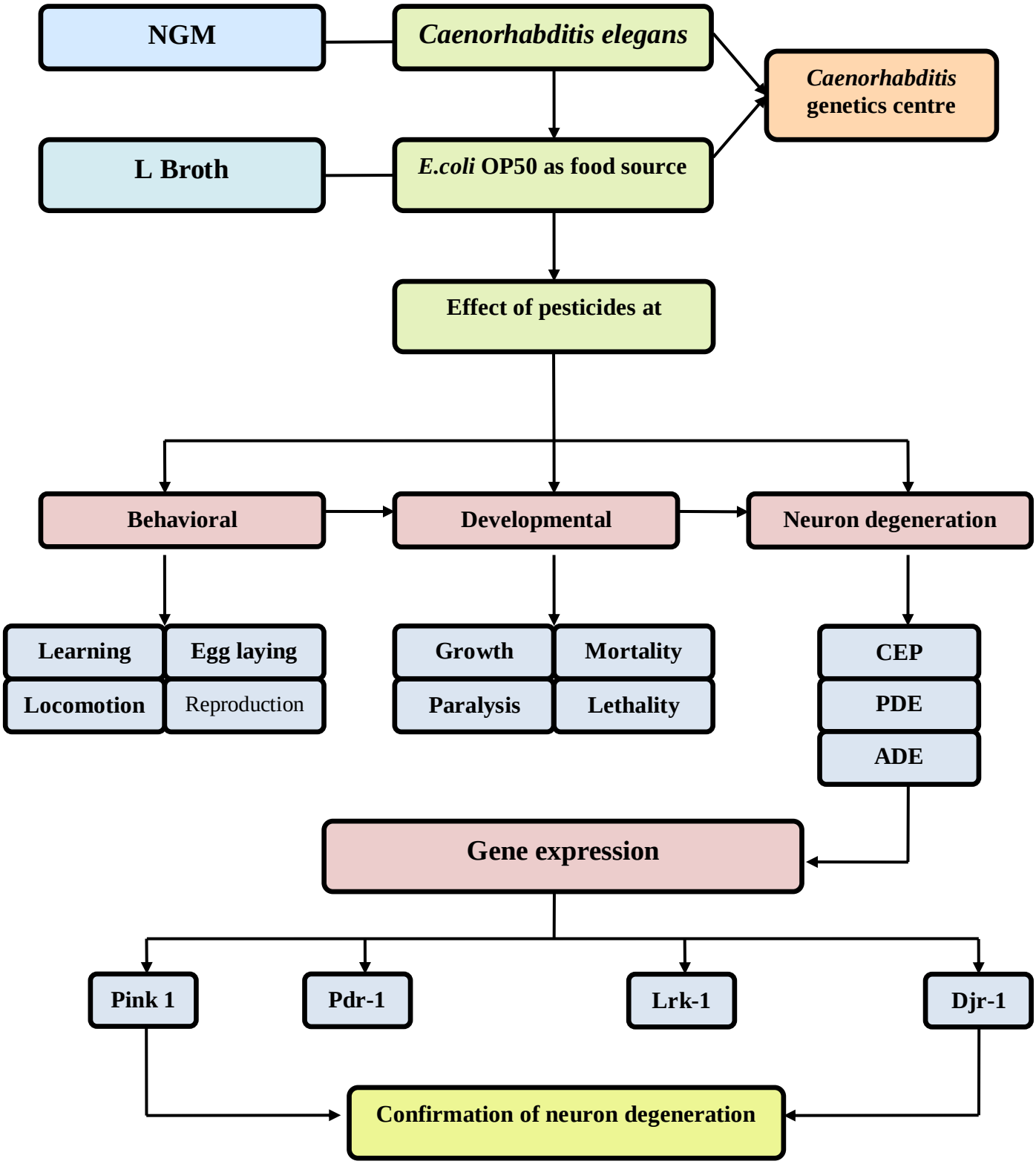
First step includes the extraction of the whole RNA content from the treated cell.

Second step includes cDNA synthesis from RNA. Further the DNA will be amplified by qualitative PCR using gene specific primers.

The extraction of the RNA will be done by using Trizol solution and precipitation with isopropyl alcohol using the methodology of Novillo *et al.,* 2005; Zhou *et al.,* 2013. After measuring the concentration of RNA using spectrophotometer the cDNA will be synthesized from the extracted RNA by using the methodology of RTPCR.doc, 2007; Reverse Transcription System, 2014; EpiScript Reverse Transcriptase, 2013. Finally the synthesized DNA will be amplified using the gene specific primers and the visualization of amplified products will be done by agarose gel electrophoresis (Springer *et al.,* 2005; Finley *et al.,* 2013).

PLAN OF WORK

Plan of work includes the research designs that how the experiments will be done in a sequential way obtains the expected outcome.



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